Methanol Chloroform Extraction of Bovine Serum and Alan Jones's Human Sepsis Serum for NMR Analysis

Biomaterials Required:
~1.0 mL serum (per sample) collected with Heparin***

Samples:
Bovine Serum (BS): non-sterile, Rockland, Cat #D200-00-0100, Lot #26415, one freeze-thaw cycle
Human sepsis serum, Jones Lab, no freeze-thaw cycles

Other reagents and solutions:

- Methanol and chloroform (reagent or HPLC grade; Fisher brand) mix 1:1 (volume) fresh in a tightly sealed (Corning screw top bottle) that has been pre-cooled (-20°C); store mixture (-20°C) so it is ice cold when ready for use
- Ice cold DI water

Other needed materials/equipment:

A. Parafilm
B. Pasteur pipets (9" borosilicate glass; Fisher brand; 22-183-632 or 13-678-20D)
C. Borosilicate glass serological filter-plugged 1mL pipets (VWR 93000-692)
D. Refrigerated centrifuge
E. Lyophilizer tubes (Fisher cat# 14-962-26D) – **Use for all areas where "culture tube" is indicated as well. Centrifuge rotor can only fit these tubes.**
F. Lyophilizer/freeze-dryer- Stringer Lab (LabConco 1L benchtop)
G. Speedvac (LabConco Centrivap Micro IR)- Stringer lab
H. "Industrial" Sharpie pen for labeling tubes
I. Disposable glass vials (Fisher brand; 03-339-25C borosilicate glass) for speedvac
J. Generic round bottom 15mL flasks (bulbs)

** Blood must be stored on ice immediately after collection and frozen at -80°C as soon as possible (<1 hour).
***The only acceptable anti-coagulant for use with sample collection or storage for this protocol is heparin. EDTA will render samples unusable. Sodium or lithium heparin work equally well.

Use glass graduated cylinders and Pasteur pipets and the glass serological pipets for Chloroform/Methanol measurements.

All samples and solutions must be kept on ice or in the refrigerator at all times!
Only use glass pasteur pipettes for transferring!

Prechill all tubes/reagents in ice or in -20 before use!

1. Thaw samples in the fridge (4°C) and mix needed reagents/solutions.
2. Label two culture tubes for each sample; put the second set of tubes in the fridge (4°C).
3. Gently invert sample tubes (DO NOT VORTEX) and remove three 10uL aliquots and add to labeled microcentrifuge tubes for BCA, Coomassie Gel and Proteomics analysis. Freeze these aliquots at -80C.
4. Add 500µL of sample to each culture tube labeled with "A." Record the actual volume that goes into each tube. This can vary if a clot has formed in the sample. Keep samples on ice.
5. Add 1mL of cold methanol:chloroform to each tube, cap and vortex thoroughly. Be careful not to let the methanol:chloroform come in contact with the tube cap. The caps are not solvent-tight.
6. Centrifuge tubes at 1300 x *g*, 4°C for 20 min.
7. Following centrifugation, transfer the supernatant and lower fluid layer to the second labeled culture tube ("L")(keep on ice). The samples may form a pellet and supernatant, or a supernatant, solid layer and lower layer of fluid. Either is acceptable, just be sure to remove all fluids leaving only the pellet (it doesn't matter of you get some of the pellet). Reserve this fluid layer in the refrigerator (4°C) or on ice (figure 1). Pellets from whole blood samples will be larger and harder than those of serum or plasma.

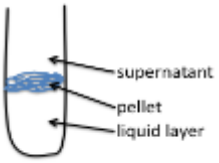

Figure 1: Representative depiction of blood or serum sample following centrifugation in step 5.

8. Resuspend the pellet of each sample in cold methanol:chloroform (0.5mL). Vigorously vortex to break up as much of the pellet as possible. Centrifuge (1300 x *g*, 4°C for 20 min).
9. Collect the supernatant (at this point, there should only be a supernatant and pellet but there may be some "floaters") and add it to the fluid layer that was reserved in step 6. Reserve pellet and any floaters.
10. Add 0.5mL of ice-cold DI water to each of the fluid tubes. Vortex well.
11. Cool the fluid for 20 minutes at -20°C. ALWAYS cool for exactly 20 minutes. If you should run over, remove tubes from the freezer re-vortex and repeat this freezing step (step 10). You don't want the aqueous phase to freeze. Ice crystals will form and precipitate in the organic phase and this will lead to erroneous results. If the sample freezes, thaw it (4°C) and repeat step 10.
12. The tubes containing the pellet can also go in the freezer (-20°C) at this point (step 10).
13. Upon removal of the tubes from the freezer, add 0.5mL of ice-cold DI water to the tubes containing pellet. Suspend the pellet by vigorously vortexing or use a spatula as needed.

14. After the 20min freezer incubation, the fluid samples will have separated into a supernatant, aqueous top layer and a protein layer (see figure 2). Add the top layer to the resuspended pellet (step 12). Do not disrupt or penetrate white protein layer. This remaining protein layer and lower liquid layer is the lipid fraction which can be capped and placed in the fridge (4°C).

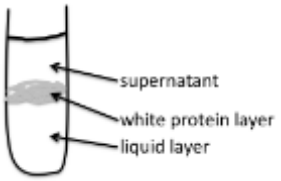

Figure 2: Representative depiction of blood or serum sample following freezer incubation in step 10.

15. Vortex the tubes containing the pellet and the newly added aqueous top layer (these are the water soluble samples) and centrifuge (1300 x *g*, 20 min, 4°C).
16. Transfer the supernatant to a lyophilizer bulb/flask. Cover with lid or parafilm.
17. Add 0.5mL ice cold DI water to pellet and resuspend as in step 10 and centrifuge (1300 x *g*, 20 min, 4°C).
18. Transfer the supernatant to the existing samples in the lyophilizer bulbs and discard the pellet.
19. Allow the water soluble fractions to sit in the lyophilizer bulbs for 5 minutes. Carefully observe each glass. If oil droplets have formed at the bottom of the glass, use a Pasteur pipet to remove them and add to the lipid fraction. Measure and record the volume in the glasses before freezing with glass serological pipet! **NOTE: Add 1mL of distilled water to each bulb to dilute out remaining methanol before freezing.**
20. Freeze the lyophilizer glasses in -80C freezer while tilting the glasses if possible (do this by placing the bulbs in the lyophilizer flask and covering each with kimwipes and placing in -80 overnight). Lyophilize for at least 24 h to ensure that methanol is removed. Do not exceed 48 h.
21. Transfer the lipid fractions to the 3.7mL borosilicate vials (measure and record volumes using serological glass pipets) and dry the lipid fractions in a speedvac at 50°C on heat + IR setting for 1h or until dry – record time for each. Cap tubes[1], wrap in parafilm and store at -20°C (can be stored for months).
22. When the water soluble samples are dry, they can be sealed with parafilm and stored (4°C) for up to a week. At the time of assay or for long-term storage, add 0.5mL of D_2O (record volume after resuspending) to each lyophilizer glass to resuspend sample. For assay, transfer each sample to an NMR tube for analysis. For long-term storage (years), transfer sample to a micro-centrifuge tube, seal with parafilm and store (-20°C). Collect another 10uL aliquot for post-extraction BCA analysis. Freeze this aliquot at -80C.
23. At the time of assay of the lipid samples, discard cap, resuspend samples in 0.6mL[2] 1:2 deuterium methanol:deuterium chloroform solution (volume to volume), cap with a new cap and centrifuge (1300 x *g*, 10 min, 4°C). Transfer supernatants to NMR tubes for assay.

[1] solvent vapors can cause degradation and failure of plastic caps
[2] for a smaller original sample (i.e., ≤ 200µL) use 500µL

A. Jones Carnitine Sepsis Serum Extract Filtration

Filtration needs to be done on all samples that display the “protein hills” upon NMR acquisition.

Currently, this has only been seen in plasma and serum samples in all species.

Materials:

1. Pall Nanosep 3K Omega Centrifugal Devices (Cat# OD003C34)
2. Deuterium oxide (Acros Organics Cat# AC426931000) – 2.5mL per sample total
3. Microcentrifuge with rotor capable of 14,000 x g force
4. Long Glass Pasteur Pipets (Wilmad LabGlass Cat# 803A)
5. 5mm 7-inch long NMR sample tubes (Wilmad LabGlass Cat# 528-PP-7)

Samples:

Sample	Pre-filter Volume (uL)	Post-filter Volume (uL)	Volume of D2O added to filtrate (uL)

Procedure:

1. Rinse filters by adding about 500uL D2O into each centrifugal device (do not add to top of tube, leave about 1cm of space at top).
2. Spin at 14,000xg for 4 minutes.
3. Repeat this wash 4-6 times with new D2O.
4. Discard all filtrate. This contains the glycerin trapped on the filters.
5. Add the entire volume of the extracted sample to the washed centrifugal device. Do not exceed 500uL. Record this volume.
6. Spin each sample at 14,000xg at 4^{o}C for 20 minutes.
7. After the spin, take only the liquid off of the top of the filter without disturbing the material on/in the filter. Gently scrape the filter with the same pipet tip. Finally, add the reserved liquid back on top of the original filter.
8. Repeat step 6 to recover more metabolites.
9. Discard filters and close centrifuge tubes.
10. Measure filtrate volume. Add D2O to end volume of 450uL.
11. To this, add 50uL of 12mM formate internal standard.

If not running NMR on same day:

12. Store samples with parafilm covering each cap at 4C if expecting to run NMR within 5 days. If longer, store at -20C.